Policy for Use of Freund's Adjuvant

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Use of Freund's Adjuvant:

Because Freund's complete adjuvant can cause inflammation, induration or necrosis, the use of Ribi adjuvant, incomplete Freund's adjuvant or other adjuvants causing less discomfort is encouraged.

Guidelines for Using Freund's Adjuvant:

If complete Freund's adjuvant is necessary, the following guidelines have been put together in order to minimize animal discomfort.

Any departure from these guidelines requires adequate scientific justification approved by the IACUC.

1. Complete Freund's adjuvant should only be used for the first antigen dose. Subsequent injections should be with incomplete Freund's.
2. Footpad injections with complete Freund's adjuvant are not acceptable.
3. Injections of complete Freund's adjuvant should be subcutaneous or intraperitoneal. Intradermal injections can cause skin ulceration and necrosis. Intramuscular injections may lead to temporary or permanent lameness. Intravenous injections have been known to produce pulmonary lipid embolism.
4. The injection containing the adjuvant should be divided into fractions so that no more than 0.1 ml is injected per site (subcutaneously) in rabbits or no more than 0.05 ml in mice.
5. The injection site shall be monitored regularly for evidence of severe inflammation, large granuloma or ulceration. The veterinary staff must be contacted if any of these symptoms occur.
6. Animals must be observed for evidence of pain, distress or infection resulting from the injection. The veterinary staff must be contacted should any of these symptoms occur.
7. The inoculum should be free of extraneous microbial contamination. Millipore filtration of the antigen prior to mixing with adjuvant is recommended.
8. Injection sites should be clean and free of debris, but need not be aseptically prepared.

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IACUC: Complete Freund's Adjuvant (CFA)

Guide for Research Use of Complete Freund's Adjuvant (CFA)

Complete Freund's Adjuvant (CFA) is widely used and considered to be the most effective adjuvant available for consistently producing high titer antibodies to diverse antigens. It is irreplaceable and vital for immunology research and antibody production at the present time. The Institutional Animal Care and Use Committee (IACUC) has developed the following recommendations to permit the continued use of CFA while maximizing antibody titer and minimizing the adverse effects of excessive inflammation, arthritis and other toxic effects.

CFA is an water-in-oil emulsion containing mycobacterial cell wall components that potentiate the humoral antibody response to injected immunogens. Adjuvant activity results from sustained release of antigen from the oily deposit and stimulation of a local immune response.

Unless specifically approved by the IACUC, antibody production using CFA at Emory University must be done using these guidelines. Requests for deviations must be scientifically justified and will be considered on a case-by-case basis by the IACUC at the time of protocol review or in response to a request for protocol modification.

Toxicity of CFA

CFA used improperly or excessively can cause undesirable side effects in animals. It may produce severe chronic local inflammation causing skin ulcerations and draining sinuses with granulomas. The oil droplet may disseminate and produce systemic granulomas and chronic wasting disease. It may also induce an autoimmune disease, especially arthritis, that is debilitating to the animal host. These adverse effects can be minimized or eliminated by adhering to the following protocol.

Guidelines for Use

1. Non-inflammatory adjuvants or adjuvants that produce less intense responses but still produce a humoral antibody response should be used if possible. (See Other Adjuvants).
2. The concentration of Mycobacteria in the CFA antigen-adjuvant emulsion should be 0.5 mg/ml or less.
3. CFA is only necessary for the initial immunization with Incomplete Freund's Adjuvant (IFA) used in subsequent immunizations.
4. The priming dose with CFA should be followed by boosters given either (1) as a response to confirmed waning antibody titers or (2) empirically once between 4-8 weeks after priming and once again 2-3 weeks after the first booster. Except for instances separate from polyclonal antibody production (i.e., specific T cell stimulation and recruitment), this regimen should produce a good titer (> 1 mg/ml serum) for immunogenic material. If additional boosters are necessary, such should be requested via a request for protocol modification.
5. The investigator should know the characteristics of the antigen and avoid factors that will excessively stimulate or inhibit the inflammatory effect. Extraneous microbial contamination, protein contaminants, pH extremes in the antigen preparation, the presence of chromatographic by-products, such as polyacrylamide gel, or chemical contamination (i.e., SDS, urea, acetic acid, solvents) may lead to a low titer of the desired antibody. Sterilization by filtration through a low binding 0.22 micron filter (i.e., cellulose acetate) should be done whenever possible. Antigen prepared by gel electrophoresis should be either (1) eluted, lyophilized, ground to a fine powder, and resuspended in sterile saline or (2) transferred to nitrocellulose paper, trimmed and cut into fine pieces.
6. The adjuvant should be injected subcutaneously in small doses (see Table 1). For optimal immunostimulation it is recommended that the injections to non-rodents be distributed over the 4 quadrants of the animal (i.e., bilaterally, on the side behind the shoulder and in front of the hind leg). Bilateral injections at the base of the tail in rats and mice gives optimal immunological response. Intradermal and intramuscular injections are discouraged and must be specifically justified. The maximum total injection dose and amount at each site is shown in Table 1.

Table 1. The Maximum Total Volume/Animal and Maximum Amount of Adjuvant-Antigen Emulsion at Each Subcutaneous Injection Site.

SPECIES	MAXIMUM TOTAL INJECTION (ML)	MAXIMUM AMOUNT (ML)/SUBCUTANEOUS SITE
Mice	0.3	0.05
Rat	0.5	0.1
Chicken	0.5	0.1
Guinea Pig	1.0	0.1
Rabbit	1.0	0.1
Goat/Sheep*	2.0	0.2
Primates **		

* Deep intramuscular injections at a maximal volume of 0.5 ml antigen-adjuvant emulsion per site is permitted in large domestic animals.

** Freund's Complete Adjuvant is not recommended for use in primates. In many cases it causes an excessive inflammatory response and would negate any TB testing in treated animals. Should an

investigator feel that he must use this adjuvant in nonhuman primates, they should confer with the attending clinical veterinarian and seek IACUC approval.

Foot Pad Immunization

Foot pad immunization of rodents or other species should not be used for routine immunization. It may be used in particular studies where isolation of a draining lymph node as primary action site is required. Foot pad immunization requires specific justification and approval by the IACUC. In these cases:

1. The use of inflammatory adjuvants is strongly discouraged;
2. only one rear foot pad can be used per experimental animal;
3. the quantity of the antigen-adjuvant emulsion injected should be kept to a minimum (i.e., 0.05 ml); and
4. the animals should be housed on soft bedding rather than screens.
5. Rabbits should not be immunized in their "foot pads," because they do not have true foot pads.

Peritoneal Exudate

Intraperitoneal administration of antigen and adjuvant is often used in rodents to obtain high titered reagent or monoclonal antibodies. The undesirable side effects of painful abdominal distention associated with development of the peritoneal exudate can be readily avoided by daily monitoring and relieving ascites pressure as appropriate. Please refer to the *IACUC Guidelines on Monoclonal Antibody Production* for methodology for this procedure.

Potential Hazards to Research Personnel

Special care must be taken to avoid parenteral exposure of personnel involved in the preparation and administration of CFA. Accidental intradermal or intramuscular inoculation of the mycobacterial-in-oil suspensions may result in tuberculin sensitization of tuberculin negative individuals and moderate to severe local, regional, or systemic hypersensitivity reactions in individuals who are sensitized to tuberculin. Persons who have had tuberculosis may develop chronic ulcerating granulomas following injection of very small amounts of CFA. Inadvertent ocular exposure can lead to blindness. The following procedures are recommended for the safe use of CFA:

1. For non-rodents, sedate or properly restraint the animal and shave the proposed injection sites prior to administration of the antigen-adjuvant.
2. Use two sterile luer-lock syringes joined by a stopcock to prepare the emulsion.
3. Wear safety glasses.

Other Adjuvants

Most adjuvants incorporate two components. One component forms a deposit to protect the antigen from catabolism. The 2 traditional methods for deposit formation are mineral oils or aluminum hydroxide precipitates (Alum). Liposomes and synthetic surfactants are alternate deposit-forming systems (Hunter et. al., 1981, Atkinson et. al., 1988). The second component is a nonspecific stimulant which acts by increasing the amount of lymphokines present. Lymphokines directly stimulate the activity of antigen processing cells, causing a local inflammatory reaction at the injection site. Heat-killed bacteria (using *Bordetella pertussis or Mycobacterium tuberculosis*) or lipopolysaccaride are used as nonspecific stimulants.

As a general suggestion, CFA should be used for weakly immunogenic compounds or for small amounts of immunogen. Several other synthetic, non-inflammatory adjuvants are available which may offer advantages in some situations. These are (1) RAS (Ribi Adjuvant System, Ribi Immunochemical Research, Inc., P.O. Box 1409, Hamilton, Montana 59840), (2) Hunter's TiterMax or TiterMax Gold (without silica) (CytRx, 154 Technology Parkway, N.W., Norcross, GA 30092) and (3) Quil A, a saponin-type, surface-active adjuvant (Accurate Chemical Scientific Corporation, Westbury, NY 11590).

Recommendations for Enhanced Antibody Production with Freund's Adjuvants

The following are *suggestions* for antigen and emulsion preparation and handling which should enhance antibody production:

1. Antisera to be used for screening bacterial expression cDNA libraries or for immunoblots are best made against denatured protein, whereas those to be used for screening cDNAs expressed in eukaryotic transfection systems or for immunoprecipitation of native-cell-synthesized structures might be best made against native protein.
2. An antigen dose range of 50-1000 micrograms is recommended for rabbits and 10-200 micrograms for a mouse.
3. As a general rule, the greatest immunogenicity is associated with the largest antigens given in the greatest quantity. Cross-linking antigen or binding to a carrier protein should be considered for nonprotein antigens and for polypeptides # 10 kD.
4. Booster doses should use half to an equivalent quantity of antigen as that which was used for priming.
5. Do not use Tris-based buffers for generating CFA or IFA emulsions. Phosphate-buffered saline is recommended for preparing antigen in solution.
6. Use glass syringes when preparing and injecting Freund's adjuvant-antigen emulsions.
7. Test for emulsion stability by extruding a small drop onto the surface of 50 ml cold water in a 100 ml beaker. An adequate emulsion will retain droplet form on the water surface.
8. Discard unused immunogen as protein denaturation will occur over time.
9. For small quantities of rare antigen, consideration should be given to direct injection of pure antigen (without adjuvant) into the spleen or lymph nodes under surgical conditions and proper anesthesia.

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CFA Subcommittee

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THE USE OF FREUND'S COMPLETE ADJUVANT

By Harold F. Stills, Jr. and Michael Q. Bailey from LAB ANIMAL April 1991 Volume 20, Number 4

Bearing the name of its creator, Freund's Complete Adjuvant (FCA) has become the most widely used means of producing immune sera in a variety of laboratory animals[1,2]. But growing concerns about pain, distress, and pathologic lesions caused by FCA have sparked debates within the scientific community about its use. Institutional Animal Care and Use Committees' (IACUC) decisions about experimental protocols that involve FCA often have little or no scientific data as a basis. A previous protocol review in this journal describes such an incident and includes a discussion about the IACUC's decision[3,4]. While many newly available adjuvants are on a par with or surpass its antibody response ability with certain antigens, FCA has the advantage of extensive background data and a wider range of antigens for which it is an effective adjuvant[5]; these factors suggest that researchers will continue to use FCA.

How an adjuvant enhances the immune response of an animal to an antigen is not clear, but two mechanisms are probably involved[5,6,7]. In the depot effect, the adjuvant protects the antigen from rapid degradation, prolonging its exposure to the host's immune system. Also, the antigen incorporates into a particulate with the deposit agent, facilitating enhanced macrophage phagocytosis. Although a variety of compounds have been useful as deposit agents, oils, which produce a water-in-oil emulsion, are most common. Paraffin oil, a non- metabolizable oil, is the deposit substance in FCA; added to this is mannide monoleate to enhance the formation of a stable emulsion. Other deposit agents include metabolizable oils like squalene-found in the RIBI system (Ribi Immunochem Research, Inc., Hamilton, MT) and TiterMax (CytRx Corp., Norcross, GA) adjuvants-alumina gel[7], ethylene vinyl acetate[8], nitrocellulose membranes[9], and biodegradable polymers[10].

At the cellular level, adjuvants enhance immune response by attracting and nonspecifically activating immune system cells. Many antigens are T-cell-dependent and require the cooperation of macrophages, T-cells, and B-cells to produce a humoral antibody response. Antigen phagocytosis by macrophages or functionally similar cells (Langerhans cells, blood monocytes, spleen, and lymph node dendritic cells) begin the humoral antibody response; the macrophages interact with helper and suppressor T-cells, thus activating B-cells and causing them to differentiate into plasma cells. The production of

lymphostimulatory compounds by the macrophage is a critical step in antigen processing and antibody response.

A variety of compounds have proven to be useful adjuvant components for stimulating and augmenting antigen processing. Killed *Mycobacteria tuberculosis* in FCA stimulates macrophage response and antigen processing. An extensively characterized *Mycobacteria* component, muramyl dipeptide, is also useful in adjuvants. Bacterial lipopolysaccharides, killed *Bordetella pertussis,* killed *Corynebacterium parvum,* and some proprietary compounds can also stimulate T-cell response. This activity has the undesirable effect of inducing an inflammatory response causing tissue destruction at the site.

Though variable on an individual animal basis, the two major factors affecting tissue reaction to FCA are injection site and volume. The three primary sites for FCA injection are intradermal, subcutaneous, and intramuscular[5,11]. In terms of antibody production and tissue destruction, all three sites have advantages and disadvantages. A controllable factor that affects the extent of tissue destruction is the volume of the antigen-FCA injection. Published recommendations on total injection volume and injection volume per site vary substantially **(Table 1).**

Intradermal Injections

Using the intradermal site creates an additional depot effect. The injection becomes localized and undispersed; Langerhans cells and macrophages come into contact with the antigen, increasing the antibody response. A disadvantage is the formation of large granulomas with ulceration at the intradermal injection site[12]. Abscesses may form at the injection site if the animal traumatizes the lesion or if the initial injection is not sterile.

For rabbits, the Canadian Council for Animal Care (CCAC) recommends the intradermal route of injection "only when the purpose is to induce cell-mediated response." [11] The CCAC-recommended maximum dose per site is 0.05 ml which agrees with Amyx[5] and the National Institutes of Health (NIH) intramural recommendations[14]. Johnston *et.al.*[13] have recently recommended 0.1 ml per site which is also recommended in laboratory immunology texts [15].

Table 1. Published recommendations for the use of Freund's Complete Adjuvant.

SOURCE	INTRADERMAL	SUBCUTANEOUS	INTRAMUSCULAR
N.I.H.[1]*	0.05 ML/N.S.	0.1 ML/N.S.	0.5 ML N.S.
C.C.A.C.[2]	0.05ML/N.S.	0.25 ML/N.S.	0.5 ML/N.S.
JOHNSON ET AL.[3]	0.1 ML/0.5 ML	0.5 ML/1.0 ML	0.25 ML/0.5ML
HARLOW & LANE[4]	0.1 ML/N.S.	.0.5 ML/N.S.	

VOLUMES LISTED: MAXIMUM PER SITE/TOTAL MAXIMUM VOLUME

N.S. - NOT SPECIFIED

*The University of Kentucky recommendation is identical to that of NIH except that intramuscular injection is not recommended for Freund's adjuvants.

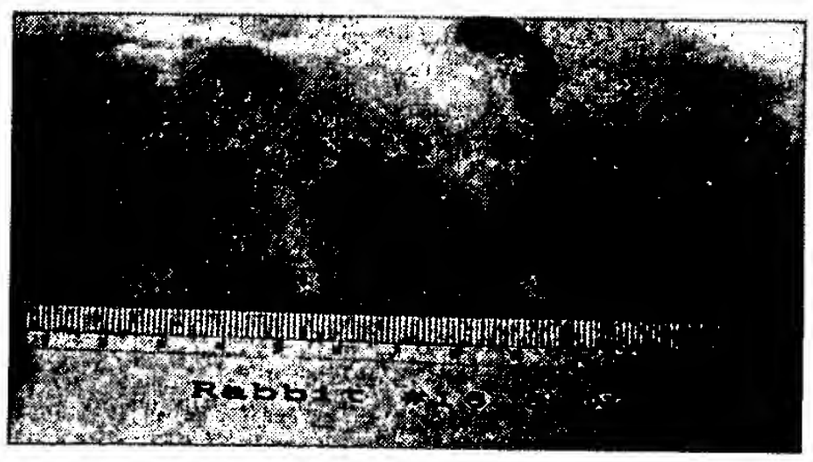
1 Grumstrup-Scott, J. and Greenhouse, D.D., eds. NIH intramural recommendations for the research use of complete Freund's adjuvant. *ILAR News;* Vol. XXX, Number 2, Spring 1988:9.

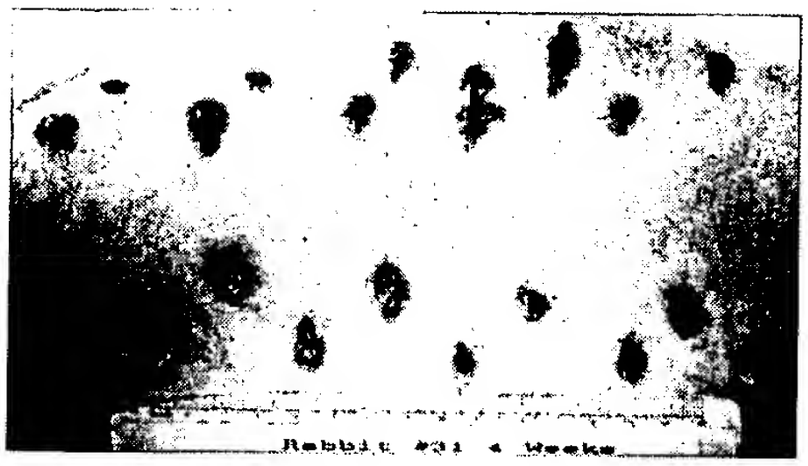
2 Canadian Council on Animal Care. *CCAC guidelines on acceptable immunological procedures.* July 5,1989. Canadian Council on Animal Care.1000-151 Slater, Ottawa, Canada Kl P 5H3.

3 Johnston, B.A., Eisen, H., and Fry, D. An evaluation of several adjuvant emulsion regimens for the production of polyclonal antisera in rabbits. *Lab Animal Sci.;* 41:15-21, 1991.

4 Harlow, E. and Lane, D. *Antibodies, A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1988

Our experience with intradermal antigen-FCA injections in rabbits indicates that the degree of tissue reaction is directly related to the injection volume per site. Granulomas averaging approximately 25 mm in diameter at 5 weeks are produced by injections of 0. 1 ml per site, while 0.05 ml and 0.025 ml per site produce granulomas with average diameters of 16 mm and 10 mm, respectively **(Figs. 1, 2 & 3).** Ulceration of the overlying skin routinely occurs at all dosages, including 0.025 ml per site. The granulomas produced by intradermal injections do not appear to be painful when palpated, and rabbits may lay in their cages with the lesions compressed against the cage side and floor.





Figures 1-3. Lesions produced in rabbits injected intradermally with a total of 0.5 ml of FCA-Mouse IgG at different volumes per site (Fig 1:5 sites, 0.1 ml per site; Fig. 2: 10 sites, 0.05 ml per site, Fig. 3: 20 sites, 0.025 ml per site). Granuloma-like lesions with ulceration of the overlying skin are present in all cases. Total lesion size is related to volume injected per site.

Injecting antigen-FCA emulsions intradermally is not easy. The viscosity of the emulsion and the nature of the injection site increases the chances that staff members will suffer accidental injection. In our experience, it is best to place the needle into the subcutaneous tissues and advance the needle point (bevel up) into the overlying dermis. With this approach, one can easily inject intradermally, and the antigen-FCA emulsion remains at the injection site **(Fig. 4).** Directly injecting the antigen-FCA emulsion intradermally often causes a portion of the emulsion to exude from the site through the needle tract.

Figure 4. An example of intradermal injection of FCA-antigen in rabbits. Proper intradermal injection is confirmed by the presence of a distinct spherical distension of the skin with overlying paleness.

Subcutaneous and Intramuscular Injections

The subcutaneous and intramuscular routes of injection are technically easier to perform and do not produce the large ulcerating granulomas associated with intradermal injections. Antibody response to subcutaneous and intramuscular antigen-FCA mixture injections is adequate in most instances. A disadvantage of the subcutaneous route is the formation of fistulous tracts extending ventrally[12], while intramuscular injections can cause muscle necrosis, fistulous tract formation, and ulceration of the overlying skin[12,13]. Lesions created by intramuscular subcutaneous injections are not easy to observe for complications; this is disadvantageous.

The recommended maximum dose per site for the subcutaneous and intramuscular routes

in rabbits varies widely, thus making it difficult to determine if an immunization protocol is acceptable. The NIH intramural recommendations[14] limit subcutaneous injections to 0.1 ml per site, while others have suggested volumes of 0.05 ml per site[12], 0.25 ml[11,13] per site and 0.5 ml per site[15]. Maximum volumes per site for intramuscular injections range from 0.25 ml[5,13] to 0.5 ml[11,15].

Our experience with subcutaneous injections of antigen-FCA emulsions is similar to Broderson's[12]. Subcutaneously injected antigen-FCA emulsions in rabbits do not remain localized and usually migrate ventrally within the subcutaneous tissues. Palpation and ultrasound examinations show that injection volumes as low as 0.05 ml per site may migrate up to 2 cm during the first week following injection. Larger volumes (0.1 ml and 0.25 ml per site) migrate further, often forming large dense "ropes" extending ventrally from the site of injection **(Fig. 5)**. Lesions are often completely absent at the injection site and are only visible if the rabbit's side is clipped.

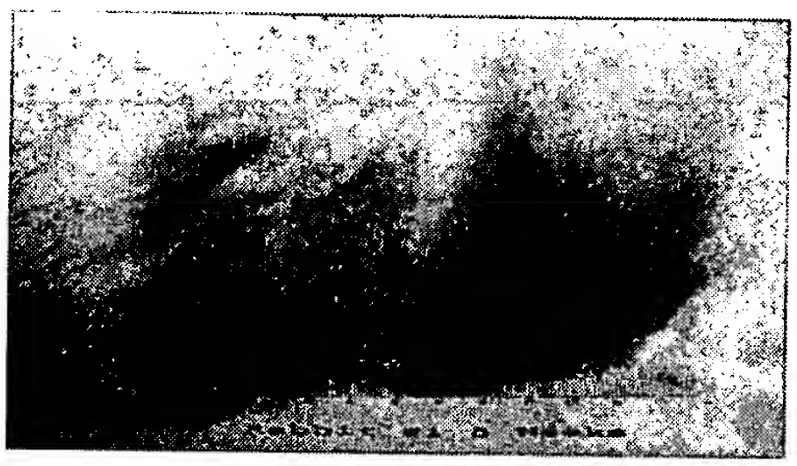


Figure 5. Large subcutaneous "rope-like" lesion present 5 weeks after the subcutaneous injection of O.25ml of FCA-Mouse IgG. The injection site was approximately 1 cm lateral of vertebral column, at the top of the figure. No reaction or thickening is present at the injection site.

Preparation and Injection of FCA Emulsions

Preparing the FCA antigen-adjuvant emulsion poses additional problems. Current methods for adjuvant-antigen emulsion preparation include commercially available double-hubbed needles and homogenizers. In all cases, the goal is to prepare a stable water-in-oil emulsion, maximizing the depot

effect of FCA. To test the completeness of emulsification, one places a drop of the antigen-FCA emulsion onto the surface of a cold water bath--a properly prepared emulsion will not spread on the surface[16] . Another characteristic of a properly prepared emulsion is high viscosity, necessitating the use of luer-lock syringes (preferably glass, since the oil reacts with the rubber plunger on disposable plastic syringes) and 23 gauge or larger needles for injection. Unless one uses 20 gauge or larger needles, injecting the emulsion should require substantial force. It is important for staff members to avoid accidental inoculation, since the resulting lesions are often severe[17].

Antigen-FCA emulsion preparation should involve sterile technique. It is necessary to clip and surgically scrub injection sites to minimize the chance of bacterial contamination. Strict adherence to sterile technique will minimize the occurrence of bacterial infections at

the injection site which can cause an acute inflammatory cell infiltrate and abscess formation; the inflammatory response is primarily due to macrophages and lymphocytes, with polymorphonuclear leukocytes being a minor component. In our experience, injection sites having an acute inflammatory reaction due to bacterial contamination appear to be quite painful; chewing, scratching, and self-mutilation of the site by the animal is not unusual.

The CCAC[11] recommends observing the injection site a minimum of three times per week for four weeks after the injection. We haven't found that this is sufficient time for intradermal injections, since most reactions appear to reach peak size between 4 and 6 weeks post-injection. Maximal subcutaneous and intramuscular lesion development is more difficult to determine, but should be similar. One must take care when evaluating subcutaneous injections since the final deposition site of the antigen-FCA emulsion is often removed from the injection site.

Pain or Distress?

It is difficult to assess the presence of pain or distress associated with adjuvant injections. Injection sites that develop an acute inflammatory cell response appear painful from the clinical point of view. Scratching, chewing, and self-mutilation of the site are all signs of pain and/or irritation. Injection sites that do not develop an acute inflammatory response are less easy to evaluate and current literature offers little information on which to base a decision. Our empirical observations, and those of other groups[13] tend to indicate that the lesions are relatively non-painful and non-pruritic.

Reports of accidental and intentional human injections provide another source of information. Chapel and August[17] reported that severe pain was present in five of nine cases of accidental human injection, and that little or no pain occurred in the other four. All of the people who suffered severe pain were positive to the tuberculin test prior to the accidental injection--this may indicate that the reaction was a result of repeat exposure to *Myco-bacteria* bacterial proteins. In an early report of immunotherapy in human cancer patients, Hughes *et.al."* discussed the occurrence of painful ulcers and abscesses in patients injected with autologous tumor extract in FCA. These reactions only occurred on the second or subsequent injection and the group attributed them to the development of sensitivity to the tuberculin antigen in the FCA.

These reports clearly illustrate that severe and painful reactions occur in tuberculin-sensitized humans injected with FCA. Other reports link repeated injections of FCA in animals to abscess and ulcer formation[13]. Repeated injection of FCA in animals is not recommended[11,13,14]. Delaying booster injections permits complete absorption of the initial injection and allows the animal to recover from the initial injection(s). In our experience, a 30-45 day delay appears to be adequate, although others recommend as few as 21 days between injections [13]. One should make booster injections in Freund's Incomplete Adjuvant (the absence of the mycobacterial proteins is the only difference), saline, or another adjuvant lacking the mycobacterial proteins.

Use of Freund's Complete Adjuvant will probably continue to be common while researchers develop and evaluate newer adjuvants. One should consider using adjuvants that produce less intense inflammatory reactions wherever possible. Trained staff members should prepare and administer Freund's Complete Adjuvant and monitor the animals

closely for undesirable reactions. Proper use of Freund's Complete Adjuvant produces exceptional immune response with a minimum of tissue damage and discomfort. Improper use may cause severe tissue damage, pain, and distress.

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